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Full Length Research Paper

Production and purification of polyclonal antibody against bovine immunoglobulins in rabbits

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Antibodies are important tools in medical researches which have led to many advances in this field. Anti-bovine immunoglobulins and its conjugate with horse radish peroxidase (HRP) is used to diagnose cows' disease by ELISA or western blotting tests. In this study, the production, purification and horse radish peroxidase (HRP) conjugation of polyclonal IgG against bovine immunoglobulins in rabbits were carried out. Three 6-month-old New Zealand White rabbits were immunized by bovine immunoglobulins in combination with Freund's adjuvant. Purified antibody (using ion-exchange chromatography) was labeled to HRP. Direct enzyme linked immunosorbent assay (ELISA) was used to determine the optimum titer and cross reactivity of HRP conjugated IgG. The purity of various IgG preparations was about 98%. The optimum dilution of prepared HRP conjugated IgG was 1:12800. This conjugated IgG has no cross reactivity with sheep and goat immunoglobulins at optimized dilution. This study showed that ion-exchange chromatography could be an appropriate method for purification of IgG antibodies.

Key words: Anti bovine immunoglobulins, horse radish peroxidase conjugation, ion-exchange chromatography, polyclonal antibody.

INTRODUCTION

Antibodies are important tools used by many investigators in their research and have led to many medical advances. Mammalian sera represent a remarkable and economical source of immunoglobulins widely used in diagnostic and therapeutic applications (Gallacher, 1993; Gathumbi et al., 2001). In biochemical and biological researches, polyclonal antibodies are routinely used as ligands for the preparation of immunoaffinity columns (Shin et al., 2001) and as coating or labeling reagents for the qualitative and quantitative determination of molecules in a variety of assays, such as enzyme linked immunosorbent assay (ELISA), double diffusion, radial immuno-diffusion, western blot and radioimmunoassays (Calabozo et al., 2001; Cheung et al., 2002; Verdoliva et al., 2000).

Horse radish peroxidase (HRP) is one of the two most widely used enzyme labels in medical diagnostics and researches applications (Handley et al., 1998). However, enzyme-labeled antibodies are used in immunoblotting, histochemical staining and ELISA techniques. They can provide an instant visual result and good sensitivity. Furthermore, using enzymes as labels offer several advantages over fluorescent and radio-labeled substances. Enzyme immunoassays reagents are more stable and do not have safety problems associated with radio isotopic labels. In addition, enzyme assays can be at least as sensitive as radioimmunoassays. Many enzyme detection methods are visual or use a standard spectrophotometer, eliminating the need for expensive, sophisticated equipment (Ali et al., 1981).

HRP conjugated IgG against bovine immunoglobulins are used in diagnosis of cows diseases by ELISA or western blotting tests. Brucellosis is one of the most prevalent infectious diseases in domesticated animals and is one of the principal health problems in Iran (Young, 1995; Feiz et al., 1978; Corbel, 1997; Hall, 1991), hence development of reagents related to diagnosis of brucellosis is of great importance. The aim of this study is the production, purification and HRP conjugation of polyclonal IgG against bovine immunoglobulins in rabbits.

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MATERIALS AND METHODS

Preparation of antigen

Blood samples were collected from ten clinically healthy cows using sterile disposable needles (1.2 - 40 mm), clarified by centrifugation (1000 g, 15 min) and diluted 1:1 with phosphate buffer saline (PBS, pH 7.2). Then equal volumes of diluted serum and saturated ammonium sulphate were mixed by slowly addition of the saturated ammonium sulphate solution with gentle stirring. After centrifugation (1000 g for 20 min), the precipitate was washed twice with 50% saturated ammonium sulphate solution. The final precipitate was dissolved in PBS followed by overnight dialysis against PBS. Protein concentration was quantified by a coomassie dye binding assay (Bradford, 1976), using bovine serum albumin (BSA) as the standard. Final protein concentration of solution adjusted to 1 mg/mL.

Immunization of rabbits with bovine immunoglobulins

Three hundred micro liters of prepared bovine immunoglobulins (1 mg/mL) in PBS was emulsified with equal volumes of Freund's complete adjuvant (Sigma) and inoculated intramuscularly (I M) into three 6-month-old New Zealand White rabbits. The rabbits were fed regular commercial diets. The second and third inoculations were performed on days 21 and 35 with Freund's incomplete adjuvant (Sigma), and the fourth inoculation was done on day 45 without any adjuvant. After the final immunization, blood samples were taken from the rabbits and production of antibody was investigated by double diffusion and ELISA tests.

This study was approved by the Regional Medical Sciences Research Ethics Committee of Tabriz University of Medical Sciences.

Purification of rabbit anti-bovine immunoglobulins

Immunized rabbits sera were collected and precipitated by 50% ammonium sulfate. After dialysis against PBS and tris-Phosphate buffer (40 tris and 25 mM phosphate, pH 8.2), ion-exchange chromatography was done on a DEAE-Sepharose fast flow (Pharmacia) in a laboratory made column at a flow rate of 0.25 mL/min. Protein concentration adjusted to 100 mg/mL and passed through the column. The column was washed in two steps using Tris-Phosphate buffer for first washing step and Tris-phosphate buffer containing100 mM NaCl for second washing step. The eluted proteins were collected in 5 mL fractions and analyzed by SDS-PAGE.

SDS-PAGE analysis

The purity of various IgG preparations was checked using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reduced conditions as described by Laemmli (Laemmli, 1970). The final concentration of polyacrylamide solution was 13%. Samples were boiled with 2% SDS for 10 min and were loaded on the electrophoresis gel. After separation, the proteins were stained with Coomassie Brilliant Blue G 250 (Blakesley and Boezi, 1977). Destaining was carried out in distilled water.

Conjugation of rabbit IgG with peroxidase

The conjugation was performed by the periodate method (Nakane and Kawaoi, 1974) with some modifications. First, 4 mg of peroxidase (Sigma) was dissolved in 0.5 mL of distilled water in dark-glass container. Then sodium periodate (Merck) was added to the solution, and the container was kept on a stirrer for 20 min at room tempe-rature. The mixture was dialyzed against acetate buffer (0.1 mM, pH 4.4) at 4°C overnight followed by addition of 10 µl of carbonate-bicarbonate buffer (0.2 M, pH 9.5). Eight milligrams of purified IgG in 1 mL of carbonate-bicarbonate buffer (10 mM, pH 9.5) was add-ed to the active enzyme, and the container was put on the stirrer. Then 150 µl of fresh sodium borohydrate solution (Merck) was added to the above solution and was kept at 4°C for 1.5 h on the stirrer. The product was then dialyzed overnight against PBS at 4°C and 1% BSA (Sigma) along with addition of 0.01% sodium mirth-iolate (Merck).

Enzyme linked immunosorbent assay (ELISA)

Direct ELISA was used to determine the titer of HRP conjugated rabbit IgG against bovine immunoglobulins. 100 µl of prepared bovine, sheep and goat immunoglobulins, which was diluted 1:100 in PBS (10 µg), was added to each well of a 96-well micro titer plate and incubated at 4°C for 24 h. The wells were washed with PBS-Tween (0.05% Tween 20) three times and blocked with 200 µl of blocking solution (PBS–0.5% Tween 20). After a washing step, 100 µl of 1:400, 1:800, 1:1600, 1:3200, 1:6400 and 1:12800 dilutions of prepared HRP conjugated anti-bovine immunoglobulins were added to each well. The reaction was developed using 100 µl of 3, 3', 5, 5'-tetramethylbenzidine (TMB) as substrate and the absorbance was determined at 450 nm after stopping the reaction by 5% sulfuric acid (Sigma).

RESULTS

Production of rabbit anti-bovine immunoglobulins

In order to survey production of antibody in rabbits and evaluating effectiveness of immunization, double diffusion and ELISA tests were performed. The titer of polyclonal anti-bovine IgG in double diffusion test was 8, which appeared as a sharp band between antigen and antibody wells. The titer of anti-bovine immunoglobulins determined by ELISA was 16000.

Purification of rabbit anti-bovine immunoglobulins

Purification of IgG rich fraction from immunized rabbit sera by ammonium sulfate precipitation followed by DEAE ion-exchange chromatography resulted in a highly pure fraction (first peak). The protein content of this fraction was 45 mg which was about one third of primary protein content (Figure 1).

SDS-PAGE analysis

Figure 2 shows the results of SDS-PAGE for determining the purity of IgG, which was purified by ion-exchange chromatography. A distinct polypeptide band with molecular weight about 50 kDa corresponding to rabbit IgG heavy chains. The diffused bands between molecular weights of 20 – 30 kDa correspond to rabbit IgG light chains. (Figure 2) The SDS-PAGE analysis showed that purification of IgG by ion-exchange chromatography re-

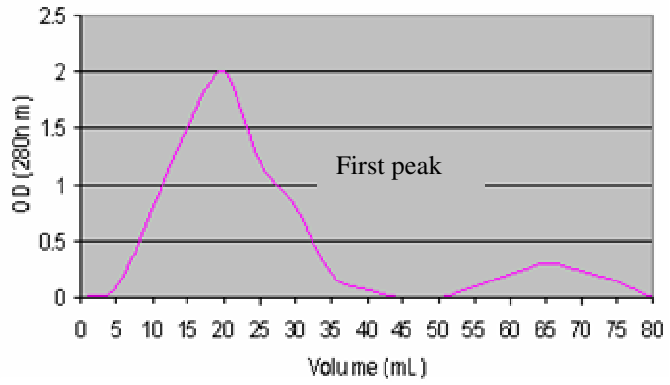

Figure 1. Chromatographic pattern of purified rabbit anti-bovine immunoglobulins by ion-exchange column with Tris-phosphate buffer, pH 8.1 (first peak) and 100 mM NaCl elution (second peak). Sample, Rabbit IgG; Matrix, DEAE Sepharose; working buffer, first step is Tris-phosphate buffer and second step is Tris-phosphate buffer +100 mM NaCl.

resulted in a highly pure product.

Enzyme linked immunosorbent assay

Direct ELISA was used to determine the optimum titer of conjugated IgG against bovine immunoglobulins produced in rabbits. The optimum dilution of prepared HRP conjugated IgG was found 1:12800. Analysis of cross reactivity with sheep and goat immunoglobulins showed that conjugated IgG has no cross reaction at optimized dilution.

DISCUSSION

In this study rabbits were immunized with bovine immunoglobulins. After multiple immunizations, blood was collected and ion exchange chromatography was used for antibody purification. To evaluate polyclonal antibody titer, designed ELISA test was applied. Because of directed evaluation of antigen-antibody complex, the test has a high degree of precision (Howard and Bethell, 2000). Determ-ining a titer of 16000 in this test indicates the high quality of the product. Thus, this antibody is highly economical and regarding the volume of 10 mL of serum taken from each rabbit at each sampling, considerable amount of anti-bovine IgGs can be obtained which meets a large portion of the requirements of the country in terms of research or educational programs. In some cases such as electron microscopy, polyclonal antibody acts better than monoclonal antibody where detection of antigen with various epitopes is the target of study, since polyclonal antibody can connect to the more connective sites resulting in better sensitivity.

The purification of immunoglobulins presents several practical complications, especially for polyclonal antibody production (Verdoliva et al., 2000). We used ion-exchange chromatography for purification of rabbit IgG polyclonal antibody. Separation and recovery of proteins from ion exchange chromatographic media are affected by factors such as buffer type and pH, length of gradient, flow rate of the mobile phase, ionic strength and nature of counter ion, and characteristic of the proteins. The selection of ideal conditions for protein purification involves changing some or all of these parameters (Tishchenko et al., 1998). This technique was well established in our laboratory for purification of IgG antibody (Baradaran et al., 2006; Javanmard et al., 2005; Majidi et al., 2005). Furthermore, ion-exchange chromatography is considered as an economical alternative to affinity and immunoaffinity chromatography. After purification step we obtained a protein with approximate purity of 98%. SDS-PAGE analysis showed that the protein with approximately 50 kDa MW was rabbit IgG heavy chains. The light chain of rabbit IgG appeared as a diffused band of 20 – 30 kDa molecular weights. It is likely that diffused band of light chain could be related to different level of deglycosilation of protein during manipulation process.

In a direct ELISA against bovine immunoglobulins (10 µg/mL), the optimum dilution of prepared HRP conjuga-

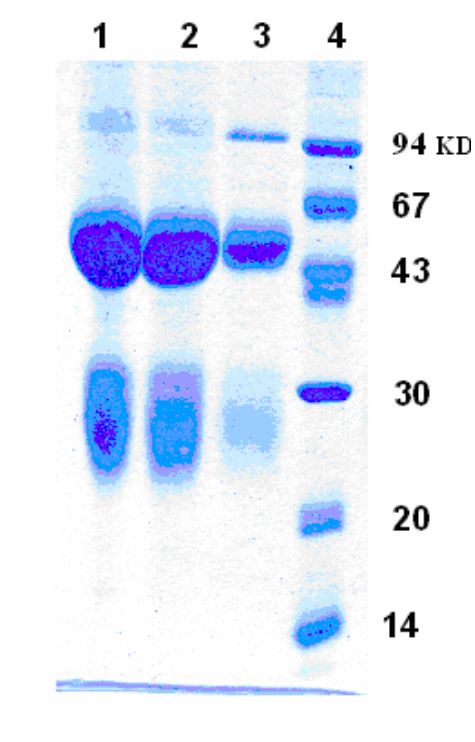

Figure 2. SDS-PAGE of rabbit anti-bovine immunoglobulins IgG, purified by ion exchange chromatography in 13% polyacrylamide gel under reduced conditions and stained with Coomassie Brilliant Blue G-250. First fraction (Lanes 1 and 2), second fraction (Lane 3) and low molecular weight markers (Lane 4).

ted IgG was 1:12800. Analysis of cross reaction with sheep and goat immunoglobulins showed that conjugated IgG has no any cross reaction at optimized dilution. Production of purified polyclonal antibody and HRP conjugated IgG against bovine immunoglobulins in rabbits could be consider another step toward Iran self sufficiency.

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